LA-UR- 02-6910

Title: Spectral Imaging and Biomedicine: New Devices, New Approaches

Author(s): Richard M. Levenson, CRI Inc.
Paul J. Cronin, CRI Inc.
Neal R. Harvey, NIS-2, LANL

Submitted to: Proceedings of the 31st IEEE Applied Imagery Pattern Recognition Workshop (AIPR 2002)

Los Alamos
NATIONAL LABORATORY

Spectral Imaging and Biomedicine: New Devices, New Approaches

Richard M. Levenson, Paul J. Cronin
CRI, Inc.
rlevenson@cri-inc.com, pcronin@cri-inc.com

Neal R. Harvey
Los Alamos National Laboratory
harve@nis.lanl.gov

Abstract

The advent of molecular medicine and new demands on pathologists to deliver prognostic and therapy-shaping analyses has created a need for enhanced imaging tools. Spectral imaging coupled with microscopy is a relatively novel and largely unexplored technology that holds out promise of satisfying, at least in part, such a need. New optical methods for spectral discrimination are being combined with powerful software approaches, often originally developed in different fields, to explore and exploit a wealth of information beyond the capabilities of conventional color-based imaging approaches.

Some of the new devices and software tools are described and illustrated here. While the results are indeed promising, it must be stressed that this field is in its infancy, and the optimal uses of this technology in the clinical arena still await definition.

1. Introduction

1.1 Multiplexing in biomedicine

The genomics revolution and the advent of truly molecular medicine have greatly increased the pressure on pathologists to provide highly detailed information on individual patient's tumors. It is no longer sufficient to arrive at an accurate diagnosis, and along with that, broad statistical predictions of how the patient might fare. Now, predictive and prescriptive information that can give precise risk estimates, and more importantly, therapy guidance, are being asked of the pathologist. New tools are needed. While much excitement is currently directed towards the use of DNA-chip-based RNA expression arrays for molecular profiling of tumors [1, 2], it may turn out that these tools will be ultimately be more useful in cancer research than in the clinic [3], for a variety of reasons, including reproducibility, cost, throughput and actual incremental utility in patient management. Traditional anatomic assessment of tumors, coupled with clinical staging, seems to perform at about the level of current expression array systems, at least in terms of prognostication. What may evolve is a system in which molecular (DNA, RNA, protein) characteristics, particularly those that qualify or disqualify patients for particular treatments, are evaluated *in situ*, in relatively intact histological specimens. This avoids the problem of extracting and, if necessary, amplifying these molecules prior to detection, a process that can destroy anatomic correlates, and may blend dissimilar histological elements into a single mixture, the so-called "Waring Blender" problem. While in-situ imaging is already the standard of care in breast cancer, in which estrogen-receptor and Her2/neu levels are evaluated using immuno-histochemistry or fluorescence in-situ hybridization (FISH), demands for these assays will continue to increase. It has been estimated that over 500 new drugs are currently under development that will require the patient to be molecularly qualified prior to treatment (D. Rimm, personal communication). An ability to perform multiplexed image-based molecular assays will be essential.

In addition to such pressing clinical demands, basic biology research as well as new drug development will require multiplexed assays. It is abundantly clear that cell-by-cell (rather than population-averaged) molecular abundance data is necessary in order to begin to understand the complex interlocking biochemical pathways that govern cellular behavior. Furthermore, even though tissue microarrays have provided the researcher with the ability to examine molecular data from 100's or 1,000's of samples at a time, these will also benefit from multiplexed imaging.

The development of novel fluorescent reagents open up new opportunities for multiplexed imaging, with the potential to detect 10's to 1,000's of different labels simultaneously (the wide range reflects different scenarios in which single or multiple hits are expected).

Finally, there are issues that affect the performance of even un-multiplexed imaging that require improvement. An example is that of autofluorescence, the unwanted emission of light by unstained tissue samples that can obscure the detection of specific fluorescent labels. Some

can transmit in a number of wavelength ranges (e.g., 400-720 nm or 850-1800 nm with bandwidths typically in the 7 to 20-nm range, although bandwidths as narrow as 0.1 nm have been achieved). Recently, a C-mount version (Fig. 1) in the visible range has been released, for use in routine imaging on standard microscopes. Such tunable filters are well suited to fluorescence-based analyses, having proved useful for multicolor fluorescence in-situ hybridization (FISH), for resolving multiple species of green fluorescent proteins (GFPs) with overlapping emission spectra, and for the identification and elimination of interfering autofluorescence. They are also well suited for use with quantum-dot-based fluorescent labeling. Image quality is good, as shown in

2.2 Agile Spectral Light Source

A novel approach for brightfield microscopy utilizes a CRI-developed spectrally agile light source (Fig. 2). This illumination system, which can replace the standard halogen lamp at the rear of most microscopes, can deliver any combination or intensity of wavelengths from 420 to 700 nm, including white light. This capability provides a number of advantages over previous techniques, enabling increased acquisition speed and faster analysis. Using three exposures with suitable polychromatic illumination functions, one can directly image a sample in CIE colorimetric space, under any desired illuminant or at any color temperature. Using matched filtering, one can obtain full information from all relevant spectral bands in a handful of exposures with optimal signal-to-noise.

3. Spectral Imaging Software

The uses of spectral imaging in pathology are still being explored and need to be matched to appropriate software tools. Relatively simple unmixing algorithms can be used to analyze samples probed for multiple molecular markers and imaged using either fluorescence or brightfield. Conventional hematoxylin-and-eosin- or Papanicolaou-stained pathology sections can have sufficient spectral content to allow the classification of cells of different lineage or to separate normal from neoplastic cells. Analysis of such specimens may succeed using spectral "signatures" and simple segmentation algorithms, but may also require more advanced analysis techniques. These can include a number of approaches pioneered for remote sensing purposes, [11] such as spectral similarity mapping, automated clustering algorithms in n-dimensions, principal component analysis, and sophisticated machine-learning systems. [12]
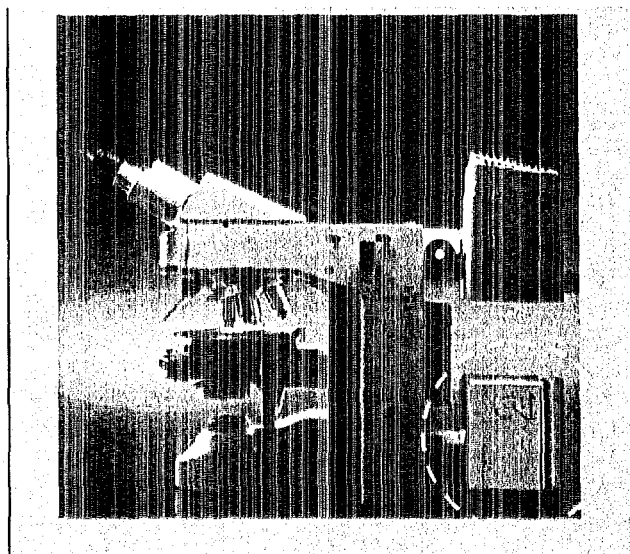
Fig. 2. Prototype SpectraLamp device. This spectrally agile source can replace the halogen lamp on most microscopes and provides multispectral illumination capabilities.

4. Applications of Spectral Imaging in Biomedicine

4.1 Fluorescence Applications: Cytogenetics

As noted above, Applied Spectral Imaging was one of the first to develop applications of spectral imaging specifically in microscopy. Their initial approach, termed "SKY" for Spectral Karyotyping, was built on their ability to resolve and quantitate at least 5 different fluorescent markers in a single image. These dyes were used to create at least 24 combinatorially encoded molecular "painting probes" (molecular reagents that would more or less uniformly bind to individual chromosomes). Combinatorial labeling refers to a strategy in which probes might be labeled with up to 3 dyes simultaneously, and in a defined dose-relationship to one another. Depending on the strategy, more than 40 different species can be distinguished.

When chromosomes from metaphase spreads were "painted" with these fluorescent markers, for the first time individual chromosomes could be identified by investigators without extensive cytogenetics training. In addition, small fragments of otherwise unidentifiable chromosomes could be detected when they were translocated out of their normal position. [5] More recently, this capability has been replicated using non-multispectral approaches that rely on multiple standard fluorescence filter cubes and combinatorial labeling strategies. [13] Both of these techniques rely on the fact that in metaphase spreads, the chromosomes are for the most part separate and non-overlapping. The latter is essential, since combinatorial labeling requires that different signals do not spatially co-localize (since having two combinatorial codes in the same pixel would generate inaccurate identifications).

absence of any exogenous fluorescent labels (Fig. 3). This procedure has the potential benefit of greatly increasing the apparent signal to noise, since the background can be appropriately subtracted without diminishing the specific signals. One study looking at multispectral imaging and protein electrophoresis obtained a 100X increase in sensitivity following a spectral background subtraction procedure. [16]

4.3 Brightfield Applications: Immunohistochemistry (IHC)

Pathologists by and large would prefer to have little to do with fluorescently labeled specimens that usually require complicated and expensive microscopes, equipped
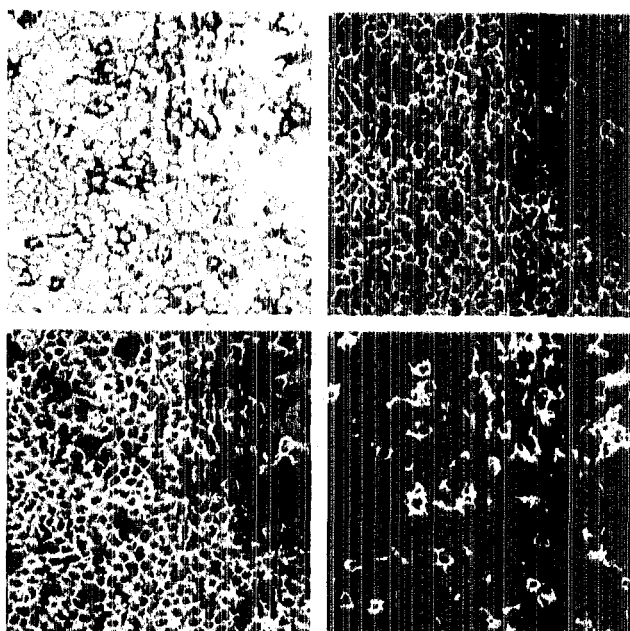
Figure 5. Spectrally resolved 2-color IHC. The sample is lymph node stained with antibodies against different cell-surface proteins. The question: are all the red-stained cells also positive for CD3 (i..e, are of the T-cell lineage). Clockwise from top left: original stained image; spectrally unmixed blue signal only; unmixed red signal only; blue (here pseudo-colored green) and red signals superimposed. The red-stained cells in the upper right quadrant are not double-stained indicating that they are not lymphocytic, but are instead, in this case, macrophages.

with appropriate dichroic mirrors and excitation and emission filters for each fluorescent species used. The specimens themselves prefer to be refrigerated, fade slowly over time and rapidly while being viewed. Having to alternate between fluorescent and brightfield viewing can be disruptive to work flow, and it's difficult to arrange for consultation with distant colleagues except through

sharing of photographic images. For all these reasons, pathologists would prefer to examine samples whose desired molecular analytes are visualized using brightfield, chromogenic signals rather than fluorescent ones. However, it is difficult to use more than one chromogen at a time, especially if a counterstain is employed, since the colors interact subtractively, so that green plus red appears dark brown rather than bright yellow, as they would in the additive realm of fluorescence. It is difficult, either by eye or using conventional RGB cameras, to resolve and quantitate overlapping colors. However, this is often quite feasible when spectral imaging is used, the key being conversion of the image stacks from transmission data to optical density data (a simple mathematical operation that makes the signals behave linearly and additively, just as if they were fluorescent in origin).

One specific application would be to examine double labeling of cell-surface markers in hematopoietic cells. This task is usually accomplished using flow cytometry, but conversion of this to a simple IHC procedure could be simple and cost-effective. An example is shown in Fig. 5.

4.4 Brightfield Applications: Spectral and Spatial Classification

One of the exciting potential applications for spectral imaging is to classify and quantitatively evaluate conventional hematoxylin- and eosin-stained pathology specimens. While preliminary efforts have indicated that these stains do display sufficient spectral heterogeneity when bound to varieties of cell and tissue types to be used for some kinds of classification, it is probable that robust classification will require combining spectral and spatial information—which is more or less what pathologists do automatically when they examine slides.

Combining such data poses a challenge, since it is not clear how and when to use spatial and spectral metrics in an optimal manner. However, some machine learning tools developed to aid in certain remote-sensing tasks appear to be well suited to the problem of automated histopathology. [17] GENIE is genetic algorithm-based program that allows a user to circle regions that should fall into one classification (for example, "cancer") and other regions into another class ("normal"). It then randomly assembles tools from a repertoire of spectral and spatial operations that maximize the differences between the two training sets. In an evolutionary process, the combinations of operators are refined and improved until the system converges onto a solution, an explicit set of mathematical steps that use both spectral and spatial information. An example of such a classifier is shown in Fig. 6, which examines performance on specimens that were not included in the training sets themselves. Results are promising, but much work remains.